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"*Endomicrobia*": Cytoplasmic Symbionts of Termite Gut Protozoa Form a Separate Phylum of Prokaryotes

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Lignocellulose digestion by wood-feeding termites depends on the mutualistic interaction of unusual, flagellate protists located in their hindgut. Most of the flagellates harbor numerous prokaryotic endosymbionts of so-far-unknown identity and function. Using a full-cycle molecular approach, we show here that the endosymbionts of the larger gut flagellates of *Reticulitermes santonensis* belong to the so-called termite group 1 (TG-1) bacteria, a group of clones previously obtained exclusively from gut homogenates of *Reticulitermes speratus* that are only distantly related to other bacteria and are considered a novel bacterial phylum based on their 16S rRNA gene sequences. Fluorescence in situ hybridization with specifically designed oligonucleotide probes confirmed that TG-1 bacteria are indeed located within the flagellate cells and demonstrated that *Trichonympha agilis* (Hypermastigida) and *Pyrsonympha vertens* (Oxymonadida) harbor phylogenetically distinct populations of symbionts (<95% sequence similarity). Transmission electron microscopy revealed that the symbionts are small, spindle-shaped cells (0.6 μm in length and 0.3 μm in diameter) surrounded by two membranes and located within the cytoplasm of their hosts. The symbionts of the two flagellates are described as candidate species in the candidate genus "*Endomicrobium*." Moreover, we provide evidence that the members of the TG-1 phylum, for which we propose the candidate name "*Endomicrobia*," are phylogenetically extremely diverse and are present in and also restricted to the guts of all lower termites and wood-feeding cockroaches of the genus *Cryptocercus*, the only insects that are in an exclusive, obligately mutualistic association with such unique cellulose-fermenting protists.

Digestive symbioses are most common among insects feeding on wood or other lignified plant materials. The most prominent example is that of wood-feeding termites (order Isoptera), where the symbiotic digestion of lignocellulose is a complex series of events involving both the host and its gut microbiota. While the events in foregut and midgut are due mainly to host activities, the digestive processes in the hindgut are largely controlled by the symbiotic gut microbiota (5, 7).

In the evolutionarily lower termites, the bulk of the hindgut volume is occupied by unicellular eukaryotes which belong to several flagellate taxa that are unique to termites (14, 37). These oxygen-sensitive protozoa, which can make up more than one-third of the body weight of their host, are essential for wood digestion and represent the major source of cellulolytic and xylanolytic activities in the hindgut (14, 16).

The gut flagellates are regularly colonized by prokaryotic epibionts attached to the surface and by endosymbionts located in the cytoplasm or in the nucleus (see, e.g., references 1, 8, and 18). Although up to 85% of the total prokaryotes in the termite gut are associated with the gut flagellates (2), only a few of the epibiotic bacteria have been identified by using molecular techniques (23, 35, 36). While the autofluorescent microorganisms within certain smaller gut flagellates are probably methanoarchaea (19, 35), the nature and identity of the endosymbionts of all larger gut flagellates (3, 26, 27) are completely obscure.

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MATERIALS AND METHODS

Sorting of flagellates and cloning of bacterial 16S rRNA genes. Termite hindguts were carefully ruptured, and a suspension of gut flagellates was prepared as described previously (29), except that the cells were not fixed with formaldehyde. Flagellates were sorted with a micropipette and washed in phosphate-buffered saline to minimize the amount of loosely attached bacteria (30). DNA was extracted from 80 to 100 flagellates of each species. 16S rRNA genes were amplified by PCR with primers 27F and 1492R and cloned into *Escherichia coli* (29).

Primer design and cloning of 16S rRNA genes of TG-1 bacteria. DNA was extracted with a bead-beating protocol (29) from 3 to 10 hindguts suspended in 800 μl of sodium phosphate buffer (120 mM, pH 8). Termite group 1 (TG-1)-specific primers, designed with the primer design function of the ARB software package (21) (version 2.5b; http://www.arb-home.de), exactly matched all existing TG-1 sequences from *Reticulitermes* spp. and had at least three mismatches (TG1-209F [5′-AATGCGTTTTGAGATGGTCCTG-3′]) or one mismatch (TG1-1325R [5′-GATTCCTACTTCATGTTG-3′]) compared to all other sequences in the ARB database. The modified PCR protocol (29) used 1 μl of the extracted DNA as a template and consisted of an initial denaturing step (3 min at 90°C), 30 cycles (30 s at 90°C, 45 s at 54°C, and 30 s at 72°C), and a final extension step (5 min at 72°C); the optimal primer annealing temperature was experimentally determined.

Sequencing and phylogenetic analysis. Clones with correct inserts were sorted by restriction fragment length polymorphism analysis (29), and the inserts of two representative clones per ribotype (identical fragments with two different restriction enzymes) were sequenced on both strands using standard primers (30) or, in the case of TG-1-specific PCR products, M13 vector primers. Sequence data were analyzed using the ARB software package (21). The new sequences were added to the ARB database and aligned by using the Fast Aligner tool implemented in ARB; alignments were checked and manually corrected where necessary.

Phylogenetic trees were calculated by using fastDNAmL, a maximum-likelihood method implemented in ARB. The stability of the branching pattern was

tested using the neighbor-joining and maximum-parsimony (DNAPARS) methods included in the PHYLIP package implemented in ARB. The reproducibility of the branching pattern was confirmed by bootstrap analysis using the maximum-parsimony algorithm and the program Seqboot in the PHYLIP package. In all phylogenetic analyses, only those positions of the alignment that were identical in at least 50% of all sequences were used.

Probe design and in situ hybridization. Oligonucleotide probes for the clones obtained from *Trichonympha agilis* (probe TG1-Ta-Rsa [5′-ACT GAC TCC CTT GCG GGT CA-3′]) and *Pyrsonympha vertens* (probe TG1-Pv-Rsa [5′-GCT AAC TCC CTT GCG AGT CA-3′]) were designed and checked for specificity by using the respective functions of the ARB software. They had three mismatches among each other and more than three mismatches with all sequences other than TG-1 clones in the ARB database (ProbeMatch) and in GenBank (BLAST). In situ hybridization of formaldehyde-fixed hindgut suspensions (30) was performed at the maximal possible formamide concentration (30%); unspecific binding of the probes was excluded by checking every sample with a nonsense probe (30). The probe information has been submitted to probeBase (http://www.microbial-ecology.de/probebase).

Transmission electron microscopy. Several worker larvae of *Reticulitermes santonensis* were dissected, and the contents of the hindgut paunch were released into 0.05 M sodium cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde. The flagellates were prefixed for 1 h, washed three times in the same buffer, and postfixed in reduced OsO_4 {a fresh 1:1 mixture of 2% OsO_4 and 3% $K_4[Fe(CN)_6]$} for 1 h on ice. After further rinses in buffer, the cells were embedded in 3% agar, dehydrated in a series of ethanol, and embedded in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate and observed by using a Philips CEM 120 Bio-Twin electron microscope.

Nucleotide sequence accession numbers. Nucleotide sequences were deposited in the GenBank database under accession numbers AY512588 to AY512590, AY572027, AY572028, and AY679171 to AY679224.

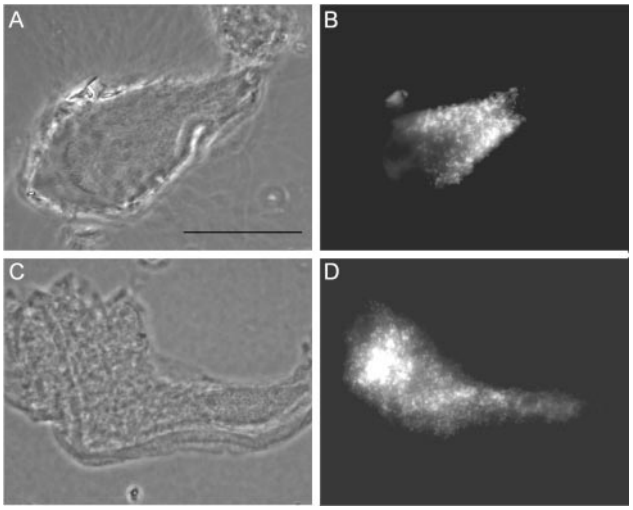

FIG. 1. Whole-cell hybridization of gut flagellates from *R. santonensis* with Cy3-labeled clone-group-specific oligonucleotide probes; each epifluorescence photomicrograph is shown next to the corresponding phase-contrast photomicrograph. (A, B) *Trichonympha agilis* hybridized with TG1-Ta-Rsa. (C, D) *P. vertens* hybridized with TG1-Pv-Rsa. Bar, 25 μm (applies to all images).

RESULTS

Cloning and sequencing. Using micropipettes, we separated 80 to 100 individuals each of *T. agilis* (Hypermastigida) and *P. vertens* (Oxymonadida), the two larger flagellate species in the termite *R. santonensis*, from the termite hindgut contents, extracted the DNA, and amplified and cloned the 16S rRNA genes of the bacteria associated with the respective flagellate. Analysis of the resulting clone libraries revealed that a single ribotype dominated among the clones from *T. agilis* (78% of the clones), and another single ribotype dominated among the clones from *P. vertens* (50% of the clones). Randomly chosen clones from each clone group had identical sequences and clustered, together with clones from *P. vertens* in the gut of *Reticulitermes flavipes*, which were obtained by using the same technique (data not shown), among the so-called termite group 1 (TG-1) bacteria (13, 24, 39).

Fluorescence in situ hybridization. The bacteria represented by the TG-1 clones obtained from the respective fractions were localized by in situ hybridization with fluorescent oligonucleotide probes. Flagellate suspensions hybridized with the bacterium-specific probe EUB 338 showed large numbers of cells within both flagellates, whereas oligonucleotide probes specifically designed for the clones from *T. agilis* or *P. vertens* hybridized only with bacterial cells within the respective flagellate (Fig. 1). They did not hybridize with any of the bacteria attached to the cell surface of the flagellates, associated with other flagellate species, or freely suspended in the hindgut fluid, confirming that the two populations of endosymbionts are specific for their respective flagellate host.

The TG-1 bacteria in *T. agilis* formed an apparently homogenous population (about 800 cells per flagellate) of small rod-shaped cells concentrated in the median portion of the cell (Fig. 1), forming a cylindrical collar around the nucleus. In *P. vertens*, the TG-1 bacteria (about 1,200 cells per flagellate) were distributed more evenly throughout the cytoplasm. Bacterial colonization of the nucleus was not observed. Approximately 10% of the *Pyrsonympha* cells also harbored a second, less-abundant morphotype of larger, rod-shaped cells (about 100 cells per flagellate) which hybridized with the EUB338 probe but not with the TG-1-specific probes. Also, these cells were distributed evenly in the cytoplasm and most likely represent the second-most abundant ribotype in the clone library from *P. vertens*. These clones (RsaPv13 and RsaPv14 [GenBank accession numbers AY572027 and AY572028]) and other clones obtained from the same flagellate species in the gut of *R. flavipes* clustered among *Bacteroidales*-related clones obtained from gut homogenates of several *Reticulitermes* species (12, 36) and were further investigated in a different context (39).

Transmission electron microscopy. Transmission electron microscopy of ultrathin sections confirmed that both *T. agilis* and *P. vertens* contained numerous prokaryotic cells which occurred exclusively in the cytoplasm of the host flagellates (Fig. 2). The uniform appearance, morphological similarity, and absence of other bacterial morphotypes in most flagellate sections were taken as strong evidence that these cells are identical to those hybridizing with the probes specific for TG-1.

The TG-1 endosymbionts of both flagellates were small rods with an average length of approximately 0.6 μm and an average diameter of approximately 0.3 μm. Their distinctly tapered cell poles, which give the cells a spindle-shaped appearance in longitudinal sections (Fig. 2A and E), are unusual. The cells appear to be surrounded by two membranes. It is not clear whether the outermost membrane represents the outer membrane of a gram-negative cell or whether it is formed by the host. The cytoplasm of the endosymbionts contained numerous ribosomes, areas of curved, parallel-packed, thin fibers (presumably DNA), and scattered electron-dense granular inclu-

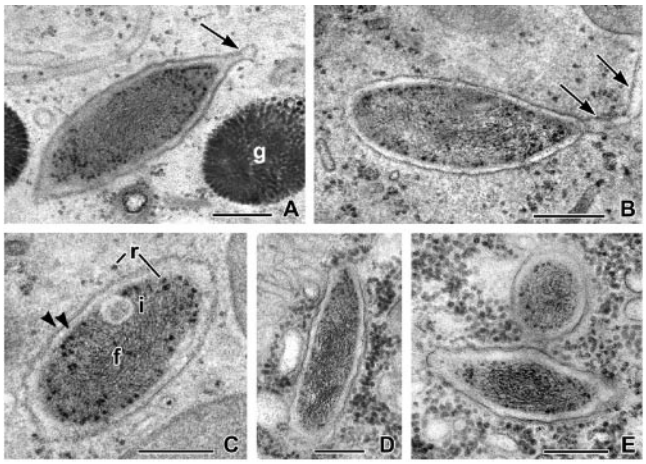

FIG. 2. Transmission electron micrographs of ultrathin sections of *T. agilis* (A to C) and *P. vertens* (D, E) showing endosymbiotic bacteria surrounded by two membranes (arrowheads in C); the outermost membrane forms tube-like elongations at the tapered cell poles (arrows in A and B). The cytoplasm of the endosymbionts contains ribosomes (r), filamentous nuclear material (f), and occasional granular inclusions (i). g, glycogen. Bars, 0.2 μm.

sions with an electron-lucent halo (complete diameter of 0.07 to 0.09 μm). In the *Pyrsonympha* endosymbionts, such inclusions were rare. Otherwise, the endosymbionts of the two flagellates appeared almost identical except that the outermost membrane of the *Trichonympha* endosymbionts formed tubular extensions of 0.05 μm in diameter extending into the cytoplasm; the maximal observed length of these structures was 0.5 μm. Such membranous extensions were never observed with the endosymbionts of *Pyrsonympha*.

Occurrence of TG-1 bacteria among other termites. Although all clones within the TG-1 phylum were amplified with standard primers, such sequences have not been retrieved from any environment other than termites of the genus *Reticulitermes* (13, 24, 39; this study). Therefore, we investigated the occurrence of TG-1 bacteria among termites from different families by amplifying a fragment of the 16S rRNA gene with newly designed TG-1-specific primers which were based on consensus regions among all TG-1 sequences available. PCR products of the expected length (approximately 1,100 bp) were obtained from DNA extracted from the hindguts of all lower termites tested and from the hindgut of the wood-feeding cockroach *Cryptocercus punctulatus* (Table 1), which represent those families that harbor oxymonadid and/or hypermastigid gut flagellates (37). No PCR product was obtained from higher termites (*Cubitermes orthognathus* and *Amitermes* sp., family Termitidae), which all lack gut flagellates.

PCR products of representatives of all families were cloned and sequenced (Table 1). A detailed phylogenetic analysis revealed that all sequences form a monophyletic group also comprising the TG-1 sequences previously obtained from *Reticulitermes* species (Fig. 3) and were only distantly related (<84% sequence identity) to their next relatives, clones obtained from activated sludge or from a contaminated aquifer (15). The isolated phylogenetic position of TG-1 bacteria relative to other phyla was supported by all algorithms and a high bootstrap value.

TABLE 1. Termite species harboring TG-1-related phylotypes[a]

Host taxon	No. of phylotypes
Rhinotermitidae	
Coptotermes formosanus	ND
Reticulitermes flavipes	ND
Reticulitermes santonensis	3
Reticulitermes speratus	4
Schedorhinotermes lamanianus	2
Termopsidae	
Zootermopsis nevadensis	1
Hodotermitidae	
Hodotermes mossambicus	3
Kalotermitidae	
Cryptotermes havilandi	ND
Cryptotermes secundus	7
Incisitermes tabogae	ND
Incisitermes marginipennis	ND
Kalotermes flavicollis	4
Neotermes castaneus	ND
Neotermes cubanus	3
Mastotermitidae	
Mastotermes darwiniensis	1
Cryptocercidae (Blattodea)	
Cryptocercus punctulatus	6

[a] In all species listed, the assays yielded PCR products with TG-1-specific primers; phylotypes represent TG-1 clone groups with less than 1.5% sequence divergence. Sequences from *R. speratus* and *R. santonensis* obtained in other studies (13, 24, 39) were included. ND, not determined.

The 16S rRNA sequence of the TG-1 bacteria in *P. vertens* from *R. santonensis* was virtually identical to the TG-1-related clones obtained from the same flagellate species in the gut of *R. flavipes* when the same procedure was used (data not shown). The closest relatives of the TG-1 bacteria in *T. agilis* were clones previously obtained from gut homogenates of the sister species *Reticulitermes speratus* (13), which also contains *T. agilis* (37). However, the next-closest relatives of the clones from these rhinotermitid species were TG-1 sequences obtained from kalotermitid and termopsid species (Fig. 3). Moreover, TG-1 sequences obtained from the same host species (e.g., *Hodotermes mossambicus* and *Kalotermes flavicollis*) fell into different clusters, which often comprised sequences from various termite families.

DISCUSSION

In this study, we demonstrated unequivocally that the endosymbiotic bacteria of the larger gut flagellates of *R. santonensis* belong to the so-called termite group 1 (TG-1), a cluster of 16S rRNA gene clones that had been retrieved previously from gut suspensions of the closely related *R. speratus* (13, 24). The first TG-1 sequences had already been reported by Ohkuma and Kudo (24) in 1996, but the novelty of the finding went unnoticed until Hugenholtz et al. pointed out that the sequences represented a new bacterial phylum (15). Although a more comprehensive clone library of the same termite species (13) indicated that TG-1 bacteria should be present in considerable numbers, their nature and identity remained completely obscure. Our results resolve the exact location of the TG-1 bacteria within their flagellate hosts and document for the first time their ultrastructure.

We also showed that TG-1 bacteria are present in, and

FIG. 3. Phylogenetic relationship of the "*Endomicrobia*" showing the position of "*Endomicrobium pyrsonymphae*" and "*Endomicrobium trichonymphae*" among the clones obtained from other termites and the wood-feeding cockroach *C. punctulatus* (Table 1). The clone designations are followed by the host species, the abbreviations for the respective family (K, Kalotermitidae; H, Hodotermitidae; R, Rhinotermitidae; M, Mastotermitidae; T, Termopsidae; C, Blattodea: Cryptocercidae), and the GenBank accession numbers. The tree is based on maximum-likelihood analysis of the 16S rRNA gene sequences of all 65 TG-1 clones and a selection of reference organisms from the next-closest related phyla. Only those base positions that were identical in more than 50% of the aligned TG-1 sequences (1,098 alignment positions) were included in the phylogenetic analyses. All marked nodes were also supported in alternate phylogenetic analyses employing maximum-parsimony and neighbor-joining algorithms and had bootstrap values (DNAPARS, 1,000 replicates) of >99% (●) and >50% (○). The bar represents 10 substitutions per 100 nucleotides.

restricted to, all insects associated with symbiotic gut flagellates. This finding supports the assumption that all TG-1 bacteria are obligate endosymbionts of termite gut flagellates and explains the uniqueness of their phylogeny and their exclusive occurrence in the hindgut of lower termites and wood-feeding cockroaches of the genus *Cryptocercus*. We therefore propose the name "*Endomicrobia*" for this lineage of uncultivated bacteria and describe the TG-1 bacteria unequivocally identified as endosymbionts of the two flagellate species as two candidate species in the candidate genus "*Endomicrobium*." Although

several environmental clones of nontermite origin had been included among the TG-1 bacteria (15), their large phylogenetic distances to the termite clones and the deep branching point (Fig. 3) indicate that they may represent yet another bacterial phylum.

Diversity of TG-1 sequences. The TG-1 sequences obtained from the various termite species are extremely diverse, but there is no direct correlation between the phylogenies of the TG-1 bacteria and the species from which they were retrieved. The TG-1 clones present in a single termite species are often polyphyletic, and the individual lineages may represent endosymbionts of different flagellates, as evidenced by the case of *T. agilis* and *P. vertens* in *R. santonensis*. Rather, the large phylogenetic distances among TG-1 bacteria from the same termite species suggest a coevolution between TG-1 endosymbionts and their flagellate hosts. However, in order to substantiate this hypothesis, it will be necessary to establish in parallel robust molecular phylogenies of the protozoa and termites involved in the tripartite symbiosis and to also take into account the rather complex distribution of certain flagellate taxa among their termite hosts (17, 37).

The evolutionary distances (maximum-likelihood distances) within the TG-1 phylum (up to 10%) are remarkable. Evolutionary distances among the *Blattabacterium* endosymbionts of *Cryptocercus* spp. and *Mastotermes darwiniensis*, which were also present in their common dictyopteran ancestor, are below 4.5% (20). Unless the molecular clock speed differs considerably among blattabacteria and TG-1 endosymbionts, the endosymbiotic event between the ancestors of flagellates and TG-1 bacteria must have occurred earlier than the divergence of termites and cockroaches in the early Cretaceous period more than 144 million years ago (34). On the other hand, the relative proximity between the TG-1 bacteria in *T. agilis* and *P. vertens* (5% sequence divergence), despite the large evolutionary distance between parabasalid and oxymonadid flagellates (10, 29), suggests a nonvertical transfer of endosymbionts among representatives of these taxa. Also, the proximity of the TG-1 sequences from *C. punctulatus* to TG-1 sequences retrieved from several termites may also add new arguments to the controversial discussion concerning an ancestral transfer of flagellate symbionts between wood-feeding termites and cockroaches (22, 33).

Ultrastructure and subcellular location. The presence of endosymbiotic bacteria in many termite gut flagellates had already been recognized quite early (1, 18). The cytoplasmic bacteria found in *Trichonympha collaris* (11) resemble the TG-1 bacteria described in the present study, although membranous extensions were not reported. Smith and Arnott (27) observed two types of endosymbionts in *P. vertens* from *R. flavipes*, a gram-positive rod and a smaller, oval, gram-negative bacterium. The latter resembles the endosymbiont of *P. vertens* from *R. santonensis* described in this study; it was found to occur free in the cytoplasm but also enclosed in vesicles, endoplasmic reticulum, and nuclear envelope. Also, Bloodgood and Fitzharris (3) reported rod-like endosymbionts in *Pyrsonympha* spp. from *R. flavipes* and *Reticulitermes tibialis* and in *Trichonympha* spp. from *R. flavipes* and the wood-feeding cockroach *C. punctulatus*. Although none of these reports contained a molecular identification, the morphology and ultrastructure of the symbionts are in agreement with the presence of TG-1 bacteria in all flagellates of these genera.

The intracellular distribution of TG-1 bacteria in the two flagellate species differs markedly (Fig. 1). In *P. vertens*, the symbionts are found throughout the cell, which has only a few internal structures (4). In *T. agilis*, they are restricted to a central cylinder which probably reflects the space occupied by the nucleus and the pronounced parabasalian body, which takes up a large portion of the cytoplasm (12). This finding is in agreement with the absence of TG-1-like bacteria from the nucleus (this study).

Possible functions of the symbionts. Phylogenetic analysis of the bacterial communities in the guts of *R. speratus* (13) and *R. santonensis* (39) indicate that TG-1 bacteria are quite abundant within the gut, representing 10 to 40% of the clones in different clone libraries. Although the spirochetes are considered the predominant bacterial phylum within the termite gut ecosystems (6), they may actually be outnumbered by TG-1 bacteria at the species level: the spirochetal community in *Reticulitermes* species is extremely diverse (6, 13), whereas TG-1 bacteria are generally represented by only a few phylotypes (13, 39; this study), probably reflecting the number of TG-1-containing flagellate species within the respective termite. Based on the abundance of *T. agilis* and *P. vertens* cells in the hindgut of *R. flavipes* (9) and the average number of TG-1 bacteria in each of these flagellates per cell (this study), we have estimated the total number of TG-1 bacteria in the hindgut as 3×10^6 to 4×10^6 cells per gut, which would represent more than half of the prokaryotic cells counted in gut homogenates of this termite (32).

In view of their abundance, the most intriguing question for future studies definitely concerns possible advantages of TG-1 endosymbionts for their flagellate hosts and their general role in hindgut metabolism. The production of cellulases and the fixation of nitrogen have been suggested as possible functions of the prokaryotic endosymbionts of gut flagellates (3), but at least the former hypothesis was already dismissed by the finding that the hypermastigote *Trichonympha* was still capable of cellulose degradation after elimination of the endosymbionts by antibiotics (38) and by the demonstration of cellulase genes in the genome of certain gut protozoa (25). However, an involvement of TG-1 bacteria in important gut processes such as nitrogen fixation, ammonium assimilation, and the provision of essential amino acids and vitamins (7) remains a feasible alternative.

Another possibility would be a function in hydrogen metabolism, e.g., by catalyzing reductive acetogenesis from CO_2 (3), but unlike *Trichonympha* species, *Pyrsonympha* species lack hydrogenosomes (4) and may not even form H_2 as a fermentation product. However, the fermentative metabolism of termite gut flagellates is poorly studied, and, especially in the case of oxymonads, further work is sorely needed. Reduced fermentation products other than hydrogen would open the possibility of hitherto-unrecognized metabolic links between the protozoa and their prokaryotic symbionts. Microinjection of radiolabeled metabolites into the hindgut of *R. flavipes* has revealed that lactate is an important intermediate in the carbon flux from polysaccharides to acetate, but the microorganisms responsible for the production and consumption of lactate remain to be identified (31).

In any case, the large numbers of endosymbionts per flagellate, their apparently ubiquitous distribution among all termites harboring gut flagellates, and the large number of flagellates in the gut already imply an important role in the tripartite symbiosis between the endosymbionts and their flagellate and termite hosts.

According to the recommendation of Stackebrandt et al. (28), who encouraged the use of the *Candidatus* concept for well-characterized but as-yet-uncultured organisms, we propose the provisional classification of the symbionts of *T. agilis* and *P. vertens* as "*Candidatus* Endomicrobium trichonymphae" and "*Candidatus* Endomicrobium pyrsonymphae" in the candidate phylum "*Endomicrobia*."

Description of the candidate phylum "*Endomicrobia*." "*Endomicrobia*" (En.do.mi.cro′bi.a. N.L. neut. n. "*Endomicrobium*", a candidate genus of bacteria; N.L. neut. pl. n. "*Endomicrobia*" phylum of the candidate genus "*Endomicrobium*") is a lineage of bacteria present in and apparently also restricted to the guts of all lower termites and wood-feeding cockroaches of the genus *Cryptocercus*. The lineage currently encompasses 33 different phylotypes with a 16S rRNA sequence divergence of more than 1.5% from 10 termite species and the wood-feeding cockroach *C. punctulatus*.

Description of the candidate genus "*Endomicrobium*." "*Endomicrobium*" (En.do.mi.cro′bi.um. pref. *endo* from G. *endon*, within; N.L. neut. n. *microbium*, microbe; N.L. neut. n. *endomicrobium*, a microbe within [a protist]) consists of small, spindle-shaped bacteria (approximately 0.6 μm in length and 0.3 μm in diameter) with distinctly tapered cell poles surrounded by two membranes. These bacteria colonize the cytoplasm of flagellate protists.

Description of the candidate species "*Endomicrobium trichonymphae*." The description of "*Endomicrobium trichonymphae*" (tri.cho.nym′phae. N.L. n. *Trichonympha*, a genus of flagellate protists; N.L. gen. n. *trichonymphae*, of *Trichonympha*, referring to the host genus) is the same as that for the genus. This species colonizes the cytoplasm of the flagellate *T. agilis*. The outermost membrane forms tubular extensions into the cytoplasm of the host. The basis of assignment was the 16S rRNA gene sequence (GenBank accession number AY512588) and hybridization with a 16S rRNA-targeted oligonucleotide probe (5′-ACT GAC TCC CTT GCG GGT CA-3′). The source was the hindgut of the termite *R. santonensis* (Feytaud); so far, it has not been cultured.

Description of the candidate species "*Endomicrobium pyrsonymphae*." The description of "*Endomicrobium pyrsonymphae*" (pyr.so.nym′phae. N.L. n. *Pyrsonympha*, a genus of flagellate protists; N.L. gen. n. *pyrsonymphae*, of *Pyrsonympha*, referring to the host genus) is the same as that for the genus. This species colonizes the cytoplasm of the flagellate *P. vertens*. The outermost membrane does not form tubular extensions. The basis of assignment was the 16S rRNA gene sequence (GenBank accession number AY512589) and hybridization with a 16S rRNA-targeted oligonucleotide probe (5′-GCT AAC TCC CTT GCG AGT CA-3′). The source was the hindgut of the termite *R. santonensis* (Feytaud); so far, it has not been cultured.

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